IMPAIRED ENDOTHELIAL FUNCTION IN PEDIATRIC HEMOGLOBIN E/β-THALASSEMIA PATIENTS WITH IRON OVERLOAD

Chalida Aphinives[1], Upa Kukongviriyapan[2], Arunee Jetsrisuparb[3], Veerapol Kukongviriyapan[4] and Nuntiya Somparn[5]

[1]Department of Radiology, [2]Department of Physiology, [3]Department of Pediatrics, [4]Department of Pharmacology, Faculty of Medicine, Khon Kaen, University, Khon Kaen; [5]Preclinical Science Division, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand

Abstract. Hemoglobin E/β-thalassemia (HbE/β-thalassemia) is the most important type of thalassemia in northeastern Thailand. Serious complications of the disease are associated with iron overload and the consequences of oxidative damage to various organs, especially the cardiovascular system. Endothelial dysfunction is an important predictor for the long-term outcome of the disease. In this study, 19 patients with HbE/β-thalassemia (aged 12.9 ± 2.8 years) and 18 healthy controls (aged 11.8 ± 1.6 years) were enrolled and their oxidant and antioxidant status was determined. Their vascular endothelial function was assessed by ultrasonographic measurement of flow-mediated dilation (FMD) of the brachial artery. The thalassemia patients were found to have higher levels of oxidative stress (based on plasma levels of malondialdehyde and protein carbonyls) and significantly reduced antioxidant levels [based on levels of glutathione (GSH) in whole blood (p<0.001)]. Thalassemia patients showed endothelial dysfunction as shown by their FMD response during reactive hyperemia (p<0.001). The degree of impaired FMD response was correlated with the age, hemoglobin levels and serum free iron levels of subjects (p<0.05). In conclusion, the FMD response was reduced in children with HbE/β-thalassemia and the degree of this reduction was correlated with the severity of anemia. FMD can be used for clinical evaluation of endothelial dysfunction, which could be an independent predictor of the cardiovascular events of thalassemia patients.

Keywords: hemoglobin E/β-thalassemia, endothelial dysfunction, flow-mediated dilatation, iron overload, oxidative stress, Thai children

INTRODUCTION

Beta-thalassemia (β-thalassemia) is a common inherited blood disorder that is caused by reduced or absent synthesis of the β-globin chain of hemoglobin (Weatherall, 1998). β-thalassemia can be co-inherited with hemoglobin E (HbE), another β-globin gene variant, resulting in a compound heterozygote state known as HbE/β-thalassemia. HbE/β-thalassemia is the most common hemoglobinopathy in Southeast Asia and the Indian subcontinent. Clinical manifestations vary among

Correspondence: Upa Kukongviriyapan, Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: +66 (0) 43 363263; Fax: +66 (0) 43 348394
E-mail: upa_ku@kku.ac.th

patients, ranging from blood transfusion-dependent thalassemia major to clinically asymptomatic individuals (Wasi *et al*, 1985; Fucharoen and Winichagoon, 1997; Fucharoen *et al*, 2004). In Thailand, the incidence of the β-thalassemia trait is about 7% throughout the country, whereas the HbE trait is about 17% in most regions, except for the northeast region where the incidence of the HbE trait is as high as 50%-70% (Fucharoen and Winichagoon, 1997; Nuntakarn *et al*, 2009; Riewpaiboon *et al*, 2010; Yamsri *et al*, 2010). Therefore, HbE/β-thalassemia is highly prevalent in the Northeast of Thailand.

Oxidative stress is evident in thalassemias (Weatherall, 1998). Reactive oxygen species (ROS) are excessively formed primarily by auto-oxidation of the globin chains, which may be enhanced by the free heme and iron overload (Shinar and Rachmilewitz, 1990; Olivieri, 1999; Rund and Rachmilewitz, 2005). The clinical features observed in HbE/β-thalassemia patients result from chronic anemia and iron overload conditions (Fucharoen and Winichagoon, 1997). Severe thalassemia is manifested as cardiovascular complications, endocrinopathies and liver disease (Olivieri, 1999; Fucharoen *et al*, 2000; Cunningham *et al*, 2004). Although iron chelation therapy improves prognosis, cardiovascular risk remains high in this population, and overt symptoms usually occur after puberty (Cunningham *et al*, 2004).

Previous studies have shown that thalassemia patients are afflicted with systemic inflammation, hemodynamic alteration and endothelial dysfunction (Aggeli *et al*, 2005; Butthep *et al*, 1995, 2004; Detchaporn *et al*, 2012). Deterioration of endothelial function is an early event in the development of cardiovascular diseases; therefore, it is a good prognostic marker for prevention and treatment of cardiovascular complications (Vita and Keaney, 2002; Verma *et al*, 2003; Halcox *et al*, 2009).

Several non-invasive techniques for evaluation of endothelial function have been developed in the past decade (Tousoulis *et al*, 2005). Flow-mediated dilation (FMD) of the brachial artery using high-resolution ultrasonography is a common and broadly applied method for evaluating endothelial function (Moens *et al*, 2005; Tousoulis *et al*, 2005). FMD is designated as an endothelium-dependent process that reflects the relaxation of a conduit artery when exposed to increased flow and, thereby, increased shear stress (Moens *et al*, 2005). Until now, endothelial function assessment using the FMD method has been done mostly on adult patients (Cheung *et al*, 2002; Aggeli *et al*, 2005; Aessopos *et al*, 2007). Therefore, the aim of the present study is to investigate the endothelium-dependent vasodilator function in children with HbE/β-thalassemia using a non-invasive FMD technique.

MATERIALS AND METHODS

Study population

The study population consisted of 19 child patients with HbE/β-thalassemia, aged 8-18 years (median age, 12 years) and 18 healthy school children, aged 9-14 years (median age, 12 years) acting as sex-matched controls. The healthy control subjects were screened by physical examination and hematologic tests. Children with HbE/β-thalassemia who attended the Pediatric Hematology Clinic in the Srinagarind Hospital, Khon Kaen University were enrolled in this study. A diagnosis of HbE/β-thalassemia was made based on hemoglobin typing and genetic analysis. These patients received

regular blood transfusions at 3-4 week intervals and were treated with iron chelation therapy (desferioxamine). The patients have received regular medical checkups in the hospital and have no overt complications such as heart disease, renal disease, liver cirrhosis or thyroid disease, as evaluated by physical examination and routine laboratory tests.

The study was approved by the Human Ethics Committee of Khon Kaen University (HE461012) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants and their parents before participating in the study. All of the parameter measurements were done on the same day before receiving a blood transfusion to avoid potential confounding effects on the assessment results. Body weight and height were measured, and body mass index was calculated. Blood samples for biochemical assessments were obtained from patients and controls after 8-hour overnight fasting.

Biochemical assays

Biochemical markers in blood samples such as hemoglobin, iron indices, lipid profiles were analysed in the Clinical Laboratory Unit of Srinagarind Hospital, Khon Kaen University. Malondialdehyde (MDA), a lipid peroxidation product in plasma, was measured using the thiobarbituric acid assay method (Somparn *et al*, 2007). Protein carbonyl, a protein oxidation marker in plasma, was assessed using dinitrophenyl-hydrazine reaction as described previously (Somparn *et al*, 2007). Accumulation of nitrate and nitrite, the oxidative products of nitric oxide (NO), was measured in plasma using the method described previously (Kukongviriyapan *et al*, 2008). Glutathione (GSH) in the blood was determined spectrophotometrically, again following a previously described method (Detchaporn *et al*, 2012).

Measurement of flow-mediated dilation of the brachial artery

FMD was accomplished according to the guidelines for ultrasound assessment of endothelium-dependent FMD of the brachial artery (Corretti *et al*, 2002). FMD was performed with a high resolution ultrasound system (Aloka Prosound SSD-3500, Ontario, CA) with the use of an 8.0 MHz linear array transducer by one experienced operator, who was blinded to the identity of the study subjects. The method of FMD is reproducible, with a coefficient of variation for repeated measurements of the brachial artery diameter of about 2%-3%.

Before FMD measurement, subjects were rested in a supine position for at least 15 minutes in an air-conditioned room with constant ambient temperature (25°C). Blood pressure and pulse rate of the subjects were measured using an automatic digital sphygmomanometer (Riester Ri-Champion, Jungingen, Germany) with a blood pressure cuff of appropriate size placed around the right upper arm for all study subjects. FMD was assessed using a high resolution ultrasound system as previously described (Sorensen *et al*, 1995; Cheung *et al*, 2002). Briefly, a non-inflated blood pressure cuff was placed around the right upper arm of the prone subject. The right brachial artery was scanned with B-mode ultrasound in longitudinal sections 2-3 cm above the elbow. Depth and gain settings were adjusted to obtain an optimal visualization of the lumen-arterial wall interface and the transducer position was kept constant during each study.

After baseline measurements of the brachial artery were recorded, the cuff was placed proximal to the section of brachial

Table 1
Comparison of demographic data, clinical parameters and hematologic profiles of patients and controls.

	Controls (n=18)	Patients (n=19)
Gender, male:female	8:10	10:09
Age (year)	11.8 ± 1.6	12.9 ± 2.8
Weight (kg)	36.9 ± 9.4	31.5 ± 8.1[a]
Height (cm)	144.1 ± 9.5	141.9 ± 15.3
BMI (kg/m^2)	17.5 ± 2.8	15.4 ± 1.9[a]
Systolic blood pressure (mmHg)	104.7 ± 11.7	107.8 ± 7.1
Diastolic blood pressure (mmHg)	57.4 ± 7.7	57.4 ± 5.2
Mean arterial pressure (mmHg)	73.1 ± 7.9	74.1 ± 4.8
Heart rate (beats/min)	83.9 ± 9.8	90.3 ± 8.8[a]
Hemoglobin (g/dl)	13.5 ± 0.6	7.5 ± 1.0[b]
Serum ferritin (ng/ml)	58.2 ± 22.5	2,323.5 ± 1,811.0[b]
Serum iron (μg/dl)	85.5 ± 37.2	144.7 ± 32.1[b]
Serum TIBC (μg/dl)	329.7 ± 49.5	181.30 ± 27.8[b]
Transferrin saturation (%)	26.6 ± 12.1	77.46 ± 9.0[b]
Total cholesterol (mg/dl)	156.1 ± 28.3	100.9 ± 22.2[b]
Triglyceride (mg/dl)	117.7 ± 50.3	137.2 ± 40.9
LDL-C (mg/dl)	80.4 ± 31.1	53.9 ± 20.0[a]
HDL-C (mg/dl)	52.0 ± 14.5	19.6 ± 4.6[b]

Data are mean ± SD. [a]p<0.05, [b]p<0.001 *vs* healthy controls. BMI, body mass index; TIBC, total iron-binding capacity; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol.

artery, inflated to 220 mmHg, and kept at the same pressure for 5 minutes to create forearm ischemia. Subsequently, the cuff was rapidly deflated, and the arterial diameter scanned continuously for 120 seconds after cuff deflation. The mean diameter of the brachial artery both pre- and post-reactive hyperemia was calculated from 3-4 cardiac cycle co-incidence with the R wave on the ECG. FMD was expressed as the percentage change of the internal diameter of the brachial artery from the baseline to post-reactive hyperemia.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical comparison between the control and patient groups was performed with the Student's *t*-test or Wilcoxon rank-sum test. Pearson correlation analysis was used to determine relationships between the different parameter measurements. All statistical analyses were performed using Stata version 7 (Stata Corp, College Station, TX). Results were considered statistically significant at a p-value <0.05.

RESULTS

The demographic characteristics, clinical parameters and hematologic profiles of the study subjects are summarized in Table 1. The average age of the controls and patients were 11.8 and 12.9 years, re-

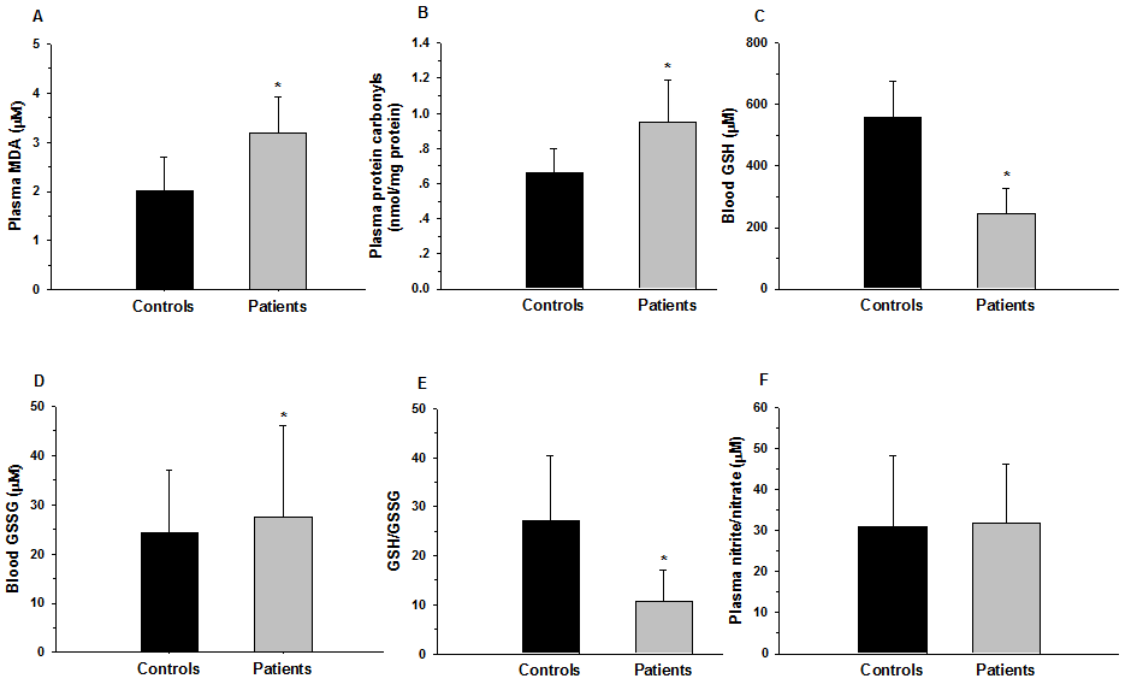

Fig 1–Oxidative stress and antioxidant markers in the patients and control subjects. Data are mean ± SD. *p< 0.001 *vs* healthy controls. MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutathione.

spectively. The HbE/β-thalassemia patients had significantly smaller body size (p<0.05; Table 1). The systolic, diastolic and mean arterial pressure did not differ between the two groups, whereas the heart rates of the patients were significantly higher than those of the controls (Table1), suggesting a compensatory mechanism for anemia in the patients. The thalassemia patients had a lower hemoglobin level in the blood (p<0.001) and higher serum iron indices, including ferritin, free iron, total iron binding capacity (TIBC) and transferrin saturation (p<0.001; Table 1). Furthermore, serum levels of total cholesterol, low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein-cholesterol (HDL-C) were significantly lower in the patients compared to the controls (p <0.001, Table 1), although the serum triglyceride levels were not different between these two groups.

Fig 1 shows the oxidant and antioxidant markers and the nitrate/nitrite levels in the patients and the control subjects. The thalassemia patients were exposed to oxidant stress conditions as shown by the higher MDA and protein carbonyls levels in plasma. Correspondingly, the blood antioxidant GSH levels and the redox ratio of the reduced GSH to GSH disulfide (GSH/GSSG) of the patients were markedly lower than those of the controls (p<0.001, Fig 1). The plasma nitrite/nitrate levels were not different between the two groups (Fig 1).

Data of the vascular endothelial function assessed by the FMD method are shown in Fig 2. The baseline brachial artery diameters were not different between the patients and controls (3.16 ± 0.27 *vs*

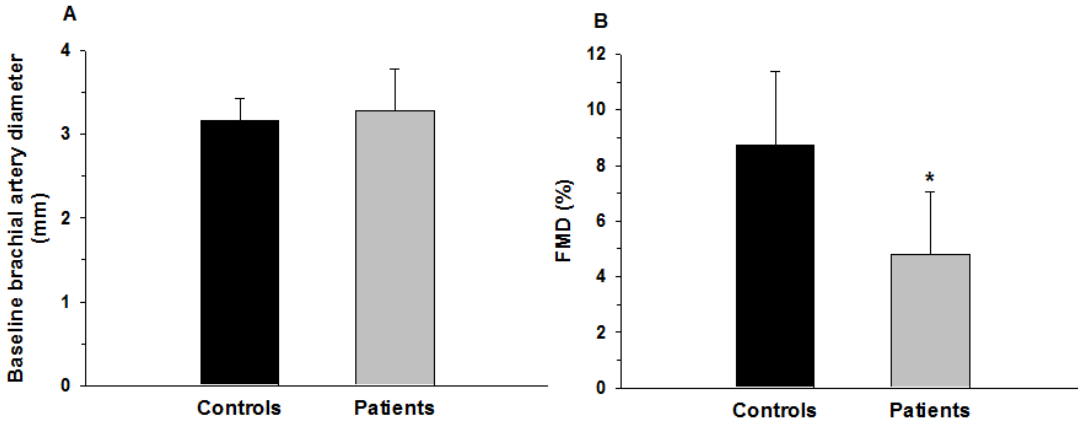

Fig 2–Brachial artery diameter at baseline and percent change of the FMD during reactive hyperemia in patients and control subjects. Data are mean ± SD. *$p<0.001$ *vs* healthy controls. FMD, flow-mediated dilation.

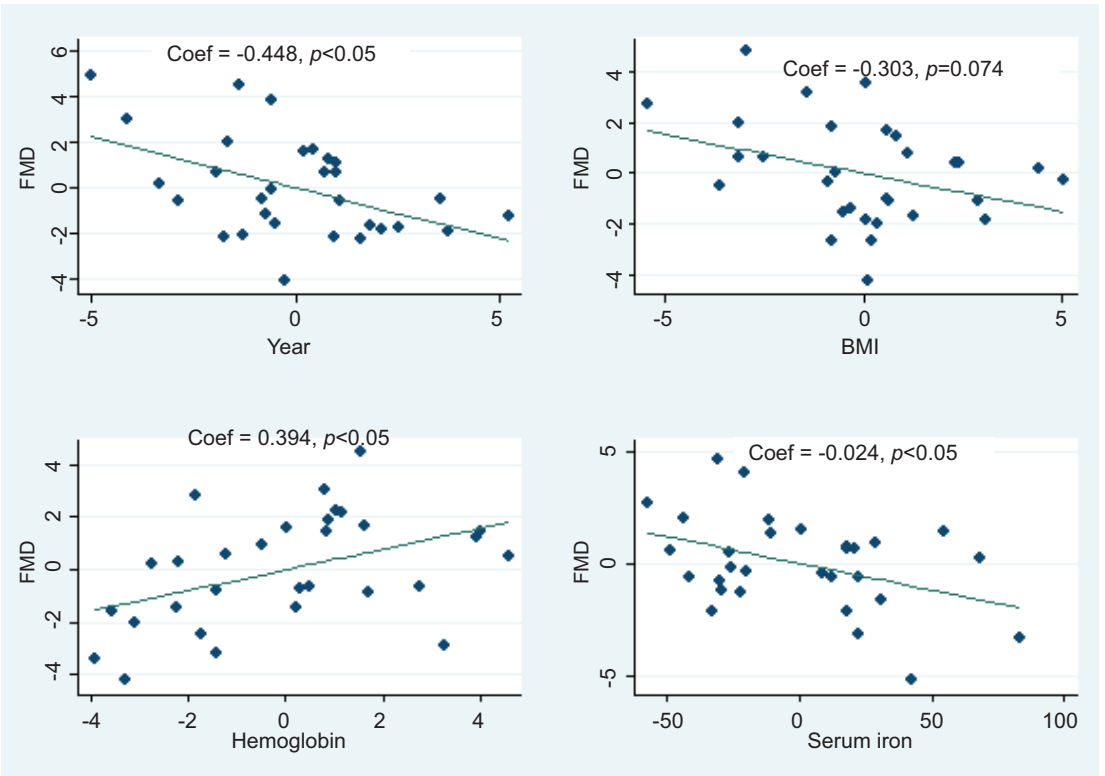

Fig 3–Partial-regression leverage plots of the individual predictors to FMD in all subjects. The plot of each predictor to the response of FMD adjusted by the other predictors including age, BMI, hemoglobin level and serum iron.

3.28 ± 0.50 mm, Fig 2). The FMD changes during reactive hyperemia, expressed as the percentage change of the brachial artery diameters from the baseline, were significantly lower in the patients compared with the controls ($p<0.001$, Fig 2). Multiple regression analysis was performed for all subjects to identify independent predictors for the change in FMD. The partial regression leverage plots are shown in Fig 3. Age, blood hemoglobin level and serum iron level were the significant predictors of the FMD ($p<0.05$, Fig 3) after adjustment with BMI and the other parameters.

DISCUSSION

The present study demonstrates vascular endothelial dysfunction, increased oxidative stress and decreased antioxidant GSH capacity in young patients with HbE/β-thalassemia. Moreover, we found that the defective vascular dilation response during FMD in pediatric thalassemia patients was correlated with the levels of hemoglobin and free iron, suggesting endothelial dysfunction is related to the severity of the disease.

Endothelial dysfunction is a hallmark of a number of diseases and is, at least in part, reversible with appropriate treatment. Early detection is therefore important for the prognosis (Verma *et al*, 2003). In thalassemia, an iron overload leads to toxicity in many organs, including the cardiovascular system, which is a leading cause of death of the patients (Hahalis *et al*, 2005). Despite improved prognosis with iron chelation therapy, cardiac mortality and morbidity remain high in the thalassemia patients (Kremastinos *et al*, 1995). Since endothelial dysfunction is an important marker of the cardiovascular disease development (Corrado *et al*, 2008; Halcox *et al*, 2009), investigation of signs of changes of cardiovascular function in young patients is of interest. In the present study, young children with thalassemia had a clear impairment of endothelial function as shown by a marked decrease in FMD during reactive hyperemia, which is assumed to be associated with reduced NO bioavailability. The most likely mechanism of reduced NO bioavailability is the interaction of NO with ROS, specifically superoxide ($O_2^{\cdot-}$). Inactivation of NO by $O_2^{\cdot-}$ contributes to oxidative stress (Moncada and Higgs, 2006). Increased oxidative stress was found in all thalassemia patients as shown by increased MDA and protein carbonyls levels, which represent lipid peroxidation and oxidative modification of proteins. Moreover, decreased cellular GSH and reduced redox ratio of GSH/GSSG, were also seen in these patients. In agreement with previous reports concerning adult patients (Livrea *et al*, 1998; Morales *et al*, 2006), decrease of total cholesterol, LDL-C and HDL-C were also noted in young thalassemia patients, confirming a depletion of cholesteryl ester resulting from a direct effect of oxidative damage.

A recent study by Suvachananonda *et al* (2013) demonstrated that the erythrocyte nitrite level, not the plasma nitrite or nitrate level, was inversely correlated with the plasma hemoglobin and lactate dehydrogenase levels in children with HbE/β-thalassemia, indicating decreased NO bioavailability is resulted from increased NO consumption in plasma and oxidative stress. In agreement with this, we also found that the plasma nitrite/nitrate levels in thalassemia patients were not decreased, but its biological activity may be diminished as a result of overproduction of ROS in the blood cells (Kukongviriyapan *et al*, 2008). It has been demonstrated that ROS generated in

thalassemia are derived mainly from the iron-catalyzed Fenton reaction and auto-oxidation of the globin chain (Shinar and Rachmilewitz, 1990). Moreover, we found that serum iron was inversely correlated with the FMD response, so that free iron is one of the predictors of endothelial dysfunction.

Chronic anemia in thalassemia patients causes a hyperdynamic circulation with increased cardiac output and decreased peripheral resistance (Aessopos *et al*, 2004).

The patients showed a low level of hemoglobin with increased heart rate, which suggests a compensatory mechanism for anemia to maintain blood pressure (Aessopos *et al*, 2004; Kukongviriyapan *et al*, 2008). Moreover, the present results showed that endothelial dysfunction is prominent in patients with clinically severe anemia and severe iron overload.

In conclusion, our study provides evidence of endothelial dysfunction in young patients with HbE/β-thalassemia. Impairment of endothelial function is associated with anemic states and the iron status of the patients. Assessment of endothelial function may be useful as a prognostic marker for the HbE/β-thalassemia patients. A study in a large number of young patients using the FMD method is needed to support this contention.

ACKNOWLEDGEMENTS

The work was supported by the Research Grant from Khon Kaen University, Thailand. The authors thank Professor Yukifumi Nawa for valuable suggestions and language editing of the manuscript.

REFERENCES

Aessopos A, Farmakis D, Hatziliami A, *et al*. Cardiac status in well-treated patients with thalassemia major. *Eur J Haematol* 2004; 73: 359-66.

Aessopos A, Farmakis D, Tsironi M, *et al*. Endothelial function and arterial stiffness in sickle-thalassemia patients. *Atherosclerosis* 2007; 191: 427-32.

Aggeli C, Antoniades C, Cosma C, *et al*. Endothelial dysfunction and inflammatory process in transfusion-dependent patients with beta-thalassemia major. *Int J Cardiol* 2005; 105: 80-4.

Butthep P, Bunyaratvej A, Funahara Y, *et al*. Alterations in vascular endothelial cell-related plasma proteins in thalassaemic patients and their correlation with clinical symptoms. *Thromb Haemost* 1995; 74: 1045-9.

Butthep P, Nuchprayoon I, Futrakul N. Endothelial injury and altered hemodynamics in thalassemia. *Clin Hemorheol Microcirc* 2004; 31: 287-93.

Cheung YF, Chan GC, Ha SY. Arterial stiffness and endothelial function in patients with beta-thalassemia major. *Circulation* 2002; 106: 2561-6.

Corrado E, Rizzo M, Coppola G, Muratori I, Carella M, Novo S. Endothelial dysfunction and carotid lesions are strong predictors of clinical events in patients with early stages of atherosclerosis: a 24-month follow-up study. *Coron Artery Dis* 2008; 19: 139-44.

Corretti MC, Anderson TJ, Benjamin EJ, *et al*. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002; 39: 257-65.

Cunningham MJ, Macklin EA, Neufeld EJ, Cohen AR. Complications of beta-thalassemia major in North America. *Blood* 2004; 104: 34-9.

Detchaporn P, Kukongviriyapan U, Prawan A, Jetsrisuparb A, Greenwald SE, Kukongviriyapan V. Altered vascular function,

arterial stiffness, and antioxidant gene responses in pediatric thalassemia patients. *Pediatr Cardiol* 2012; 33: 1054-60.

Fucharoen S, Ketvichit P, Pootrakul P, Siritanaratkul N, Piankijagum A, Wasi P. Clinical manifestation of beta-thalassemia/hemoglobin E disease. *J Pediatr Hematol Oncol* 2000; 22: 552-7.

Fucharoen G, Sanchaisuriya K, Sae-ung N, Dangwibul S, Fucharoen S. A simplified screening strategy for thalassaemia and haemoglobin E in rural communities in South-East Asia. *Bull World Health Organ* 2004; 82: 364-72.

Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia: molecular biology and clinical medicine. *Hemoglobin* 1997; 21: 299-319.

Hahalis G, Alexopoulos D, Kremastinos DT, Zoumbos NC. Heart failure in beta-thalassemia syndromes: a decade of progress. *Am J Med* 2005; 118: 957-67.

Halcox JP, Donald AE, Ellins E, *et al*. Endothelial function predicts progression of carotid intima-media thickness. *Circulation* 2009; 119: 1005-12.

Kremastinos DT, Tiniakos G, Theodorakis GN, Katritsis DG, Toutouzas PK. Myocarditis in beta-thalassemia major. A cause of heart failure. *Circulation* 1995; 91: 66-71.

Kukongviriyapan V, Somparn N, Senggunprai L, Prawan A, Kukongviriyapan U, Jetsrisuparb A. Endothelial dysfunction and oxidant status in pediatric patients with hemoglobin E-beta thalassemia. *Pediatr Cardiol* 2008; 29: 130-5.

Livrea MA, Tesoriere L, Maggio A, D'Arpa D, Pintaudi AM, Pedone E. Oxidative modification of low-density lipoprotein and atherogenetic risk in beta-thalassemia. *Blood* 1998; 92: 3936-42.

Moens AL, Goovaerts I, Claeys MJ, Vrints CJ. Flow-mediated vasodilation: a diagnostic instrument, or an experimental tool? *Chest* 2005; 127: 2254-63.

Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol* 2006; 147 (suppl 1): S193-201.

Morales NP, Charlermchoung C, Luechapudiporn R, Yamanont P, Fucharoen S, Chantharaksri U. Lipid fluidity at different regions in LDL and HDL of beta-thalassemia/Hb E patients. *Biochem Biophys Res Commun* 2006; 350: 698-703.

Nuntakarn L, Fucharoen S, Fucharoen G, Sanchaisuriya K, Jetsrisuparb A, Wiangnon S. Molecular, hematological and clinical aspects of thalassemia major and thalassemia intermedia associated with Hb E-beta-thalassemia in Northeast Thailand. *Blood Cells Mol Dis* 2009; 42: 32-5.

Olivieri NF. The beta-thalassemias. *N Engl J Med* 1999; 341: 99-109.

Riewpaiboon A, Nuchprayoon I, Torcharus K, Indaratna K, Thavorncharoensap M, Ubol BO. Economic burden of beta-thalassemia/Hb E and beta-thalassemia major in Thai children. *BMC Res Notes* 2010; 3: 29.

Rund D, Rachmilewitz E. Beta-thalassemia. *N Engl J Med* 2005; 353: 1135-46.

Shinar E, Rachmilewitz EA. Oxidative denaturation of red blood cells in thalassemia. *Semin Hematol* 1990; 27: 70-82.

Somparn N, Kukongviriyapan U, Tassaneeyakul W, Jetsrisuparb A, Kukongviriyapan V. Modification of CYP2E1 and CYP3A4 activities in hemoglobin E-beta thalassemia patients. *Eur J Clin Pharmacol* 2007; 63: 43-50.

Sorensen KE, Celermajer DS, Spiegelhalter DJ, *et al*. Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. *Br Heart J* 1995; 74: 247-53.

Suvachananonda T, Wankham A, Srihirun S, *et al*. Decreased nitrite levels in erythrocytes of children with beta-thalassemia/hemoglobin E. *Nitric Oxide* 2013; 33: 1-5.

Tousoulis D, Antoniades C, Stefanadis C. Evaluating endothelial function in humans: a guide to invasive and non-invasive techniques. *Heart* 2005; 91: 553-8.

Verma S, Buchanan MR, Anderson TJ. Endothelial function testing as a biomarker of vascular disease. *Circulation* 2003; 108: 2054-9.

Vita JA, Keaney JF, Jr. Endothelial function: a barometer for cardiovascular risk? *Circulation* 2002; 106: 640-2.

Wasi P, Pootrakul P, Fucharoen S, Winichagoon P, Wilairat P, Promboon A. Thalassemia in Southeast Asia: determination of different degrees of severity of anemia in thalassemia. *Ann NY Acad Sci* 1985; 445: 119-26.

Weatherall DJ. Pathophysiology of thalassaemia. *Baillieres Clin Haematol* 1998; 11: 127-46.

Yamsri S, Sanchaisuriya K, Fucharoen G, Sae-Ung N, Ratanasiri T, Fucharoen S. Prevention of severe thalassemia in northeast Thailand: 16 years of experience at a single university center. *Prenat Diagn* 2010; 30: 540-6.